

A physiological role of the adenosine A₃ receptor: Sustained cardioprotection

BRUCE T. LIANG*[†] AND KENNETH A. JACOBSON[‡]

*Department of Medicine, Cardiovascular Division, University of Pennsylvania Medical Center, Philadelphia, PA 19104; and [‡]Molecular Recognition Section, Laboratory of Bioorganic Chemistry, National Institute of Diabetes, Digestive and Kidney Disease, National Institutes of Health, Bethesda, MD 20892

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ABSTRACT Adenosine released during cardiac ischemia exerts a potent, protective effect in the heart. A newly recognized adenosine receptor, the A₃ subtype, is expressed on the cardiac ventricular cell, and its activation protects the ventricular heart cell against injury during a subsequent exposure to ischemia. A cultured chicken ventricular myocyte model was used to investigate the cardioprotective role of a novel adenosine A₃ receptor. The protection mediated by prior activation of A₃ receptors exhibits a significantly longer duration than that produced by activation of the adenosine A₁ receptor. Prior exposure of the myocytes to brief ischemia also protected them against injury sustained during a subsequent exposure to prolonged ischemia. The adenosine A₃ receptor-selective antagonist 3-ethyl 5-benzyl-2-methyl-6-phenyl-4-phenylethynyl-1,4-(±)-dihydropyridine-3,5-dicarboxylate (MRS1191) caused a biphasic inhibition of the protective effect of the brief ischemia. The concomitant presence of the A₁ receptor antagonist 8-cyclopentyl-1,3-dipropylxanthine (DPCPX) converted the MRS1191-induced dose inhibition curve to a monophasic one. The combined presence of both antagonists abolished the protective effect induced by the brief ischemia. Thus, activation of both A₁ and A₃ receptors is required to mediate the cardioprotective effect of the brief ischemia. Cardiac atrial cells lack native A₃ receptors and exhibit a shorter duration of cardioprotection than do ventricular cells. Transfection of atrial cells with cDNA encoding the human adenosine A₃ receptor causes a sustained A₃ agonist-mediated cardioprotection. The study indicates that cardiac adenosine A₃ receptor mediates a sustained cardioprotective function and represents a new cardiac therapeutic target.

Adenosine is released in large amounts during myocardial ischemia and can exert potent, protective effects in the heart (1). Thus, adenosine released during a brief ischemic episode can protect the heart against injury sustained during a subsequent period of prolonged ischemia, causing a reduction in the infarct size (2–5). A brief prior exposure of the heart to adenosine, instead of brief ischemia, can induce a similar protective effect. The mechanism by which adenosine causes the cardioprotective effect is incompletely understood. Although both A₁ and A₃ receptors can mediate cardioprotection (5–8), the specific protective function mediated by each receptor has not been delineated. Each receptor may serve a distinct role in cardiac tissue.

A cultured ventricular cell model has been developed (5, 9, 10) that exhibits adenosine-mediated cardioprotection, characteristic of that found in the intact heart (4, 6–8, 11, 12). Exposure of the cardiac cells to hypoxia in glucose-free medium has been used to simulate ischemia (5, 9, 10). Aden-

osine released during a 5-min exposure to simulated ischemia protected the cardiac cells against injury sustained during a second 90-min period of ischemia (5, 9). Both A₁ and A₃ receptors coexist on these cardiac ventricular cells. Prior activation of either receptor with a selective agonist can protect the cardiac cells during a subsequent period of prolonged ischemia (5).

The presence of functional A₁ and A₃ receptors capable of mediating cardioprotection in both the cardiac cell and the intact heart models suggests that the cell model represents a useful system to delineate the specific physiological role of each receptor in mediating the protection. Further, a number of advantages of the cardiac cell model enabled determination and characterization of the physiological function(s) of each adenosine receptor. First, the cultures contain predominantly cardiac cells and are devoid of vascular, circulating blood cells or neurons. Potential confounding effects arising from activation of adenosine receptors on these other cells were avoided. Second, the spontaneously beating nature of these cardiac cells suggests that they likely had metabolic requirements more akin to those of beating cardiac cells in the intact heart. Third, the extent of cardioprotection can be readily quantitated. The objective of the present study was to determine the role of adenosine A₁ and A₃ receptors in the protection achieved by the brief initial ischemia and to investigate whether the cardioprotective function mediated by the A₁ receptor differs from that mediated by the A₃ subtype.

MATERIALS AND METHODS

Materials. The adenosine analogs 2-chloro-N⁶-cyclopentyladenosine (CCPA) and 8-cyclopentyl-1,3-dipropylxanthine (DPCPX) were from Research Biochemicals (Natick, MA). 2-Chloro-N⁶-(3-iodobenzyl)adenosine-5'-N-methyluronamide (Cl-IB-MECA) and 3-ethyl 5-benzyl-2-methyl-6-phenyl-4-phenylethynyl-1,4-(±)-dihydropyridine-3,5-dicarboxylate (MRS1191) were synthesized as described (13, 14). Full-length cDNA encoding the human adenosine A₃ receptor was kindly provided by Peter Schofield (Garvan Medical Institute, Sydney, Australia). Adenosine was obtained from Sigma. The vector pcDNA3 was obtained from Invitrogen. Embryonated chicken eggs were from SPAFAS (Norwich, CT).

Preparation of Cardiac Myocyte Model of Simulation of Ischemia. Atrial and ventricular cells were cultured from chicken embryos 14 days *in ovo* and maintained in culture as described previously (9, 10, 15). All experiments were performed on day 3 in culture, at which time the medium was changed to a Hepes-buffered medium containing 139 mM

Abbreviations: CCPA, 2-chloro-N⁶-cyclopentyladenosine; DPCPX, 8-cyclopentyl-1,3-dipropylxanthine; MRS1191, 3-ethyl 5-benzyl-2-methyl-6-phenyl-4-phenylethynyl-1,4-(±)-dihydropyridine-3,5-dicarboxylate; Cl-IB-MECA, 2-chloro-N⁶-(3-iodobenzyl)adenosine-5'-N-methyluronamide; CK, creatine kinase.

[†]To whom reprint requests should be addressed at: 504 Johnson Pavilion, University of Pennsylvania Medical Center, 3610 Hamilton Walk, Philadelphia, PA 19104. e-mail: liangb@mail.med.upenn.edu.

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NaCl, 4.7 mM KCl, 0.5 mM MgCl₂, 0.9 mM CaCl₂, 5 mM Hepes (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid) and 2% fetal bovine serum, pH 7.4, 37°C, before exposing the myocytes to simulated ischemia. Simulated ischemia was induced by exposing the myocytes to 90 min of hypoxia and glucose deprivation in a hypoxic incubator (NuAire) where O₂ was replaced by N₂ as described previously (9, 10). The extent of myocyte injury was determined at the end of the 90-min ischemia, at which time myocytes were taken out of the hypoxic incubator and reexposed to room air (normal percentage of O₂). Aliquots of the medium then were obtained for creatine kinase activity measurement, which is followed by quantitation of the number of viable cells, as determined by the ability to exclude trypan blue (5). Measurement of the basal level of cell injury was made after parallel incubation of control cells under normal percentage of O₂. The extent of ischemia-induced injury was determined quantitatively by the percentages of cells killed and by the amount of creatine kinase (CK) released into the medium according to a previously described method (9). The amount of CK was measured as enzyme activity (unit/mg), and increases in CK activity above the control level were determined. The percentage of cells killed was calculated as the number of cells obtained from the control group (representing cells not subjected to any hypoxia or drug treatment) minus the number of cells from the treatment group divided by number of cells in control group multiplied by 100%.

Gene Transfer into Cardiac Myocytes. Cardiac atrial myocytes were transfected with pcDNA3 or with pcDNA3 containing the full-length cDNA encoding the human adenosine A₃ receptor (pcDNA3/hA₃R) (16) by using a newly modified calcium phosphate precipitates method (17, 18). Cardiac atrial myocytes were maintained in culture for 24 hr before being exposed to the calcium phosphate/DNA precipitates for 6 hr at 37°C. Media were replaced with fresh growth media after two washes, and the myocytes were cultured for an additional 48 hr. The efficiency of gene transfer was correlated with the formation of coarse precipitates produced by increasing the calcium phosphate concentration in the transfection cocktail.

Expression of Human Adenosine A₃ Receptor as a Functional Protein. Expression of the human adenosine A₃ receptor cDNA as a functional protein was assayed 48 hr after the transfection procedure. Its expression was determined by the ability of transfected human adenosine A₃ receptor to mediate inhibition of isoproterenol-stimulated adenylyl cyclase. An increased A₃ receptor agonist-mediated inhibition of isoproterenol-stimulated cyclic AMP accumulation in myocytes transfected with the human adenosine A₃ receptor cDNA indicated expression of the exogenous cDNA as a functional A₃ receptor. Adenosine receptor-mediated inhibition of isoproterenol-stimulated cyclic AMP accumulation was determined as described previously (5).

RESULTS

Delineation of the Protection by A₁ and A₃ Receptors: Use of Selective Agonists and Antagonists. To definitively establish that the effects of A₁ and A₃ agonists are mediated by each receptor respectively, receptor-selective antagonists that have been characterized in these cultured chicken cardiac cells (5, 10) were employed. The adenosine A₁ receptor-selective antagonist 8-cyclopentyltheophylline (CPT) blocked the protective effect of the A₁ agonist CCPA while having virtually no effect on the cardioprotection elicited by the A₃-selective agonist Cl-IB-MECA (Fig. 1). These data, similar to those obtained with other A₁ receptor-selective antagonists, DPCPX or N⁶-cyclopentyl-9-methyladenine (N-0840) (data not shown), indicate that the protective effect induced by CCPA is mediated by the A₁ but not the A₃ receptor. To determine whether the protection afforded by the A₃ agonist is mediated

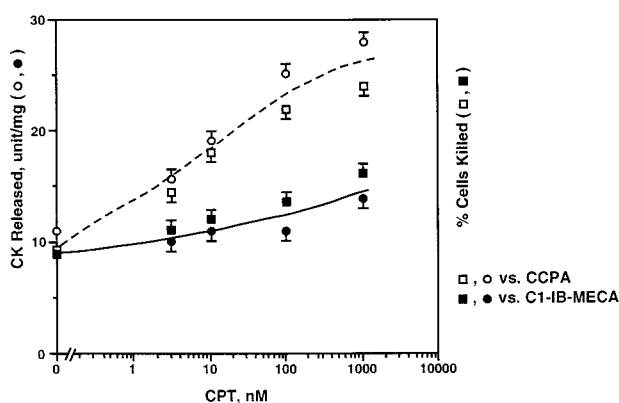


Fig. 1. Effect of 8-cyclopentyltheophylline on the CCPA- and Cl-IB-MECA-induced cardioprotective effect. Cultured ventricular cells were prepared and exposed to either CCPA (10 nM) or Cl-IB-MECA (10 nM) in the presence and absence of varying concentrations of the adenosine A₁ receptor antagonist 8-cyclopentyltheophylline. Media were changed to that lacking agonist or antagonist. Cells were then exposed to room air at 37°C for 10 min before being exposed to simulated ischemia for 90 min. The percentage of cells killed and the amount of creatine kinase released were determined after the 90-min ischemic period. Data represented the means and standard errors of four experiments.

by the A₃ receptor, antagonists selective at the A₃ receptor (10, 13, 14) were used. The antagonist MRS1191, previously shown to be selective at the adenosine A₃ receptor in these chicken cardiac cells (10), blocked the Cl-IB-MECA-elicited cardioprotection whereas it had minimal effect on the CCPA-induced protective effect (Fig. 2). Similar data were obtained with another A₃ selective antagonist, 3,5-diethyl 2-methyl-6-phenyl-4-[2-phenyl-(E)-vinyl]-1,4-(±)-dihydropyridine-3,5-dicarboxylate (MRS1097). Thus, receptor-selective agonists and antagonists can separate the cardioprotective effect mediated by the A₁ receptor from that mediated by the A₃ receptor.

Role of Adenosine A₁ and A₃ Receptors in Mediating the Cardioprotection by the Brief Ischemia. Although prior activation of either receptor can mimic the protection induced by the brief ischemia, it is not known whether both A₁ and A₃ receptors actually mediate the protective effect of endogenous adenosine released during the brief ischemia. Defining the selectivity of antagonist at each receptor subtype enabled examination of this question. Various concentrations of the A₃ antagonist MRS1191 were included individually during the brief ischemia. MRS1191 abolished the protective effect of brief ischemia in a biphasic manner (Fig. 3). The first phase of the curve, occurring at low concentrations of the drug (termed the high-affinity phase), likely was mediated by the antagonistic effect of MRS1191 at the cardiac A₃ receptor; the second phase, occurring at the high concentrations of the drug, is likely a result of its antagonist effect at the cardiac A₁ receptor. To provide further evidence for this notion, the A₁ receptor-selective antagonist DPCPX was included with MRS1191 during the brief ischemia. Concomitant presence of DPCPX (1 μM) converted the MRS1191-induced dose inhibition to a monophasic curve, similar to the high-affinity phase of the biphasic curve. The percentage of cardiac cells killed in the concomitant presence of 1, 10, 30, or 100 nM DPCPX was significantly greater than that obtained in the absence of DPCPX ($P < 0.05$, *t* test). Previous findings showed that DPCPX was able to cause only a partial, dose-dependent inhibition of the protective effect of brief ischemia (5). However, the combined presence of both antagonists caused a complete abolition of the protective effect induced by the brief ischemia (Fig. 3). Similarly, another A₃ receptor antagonist, MRS1097, in the presence of a fixed concentration of DPCPX,

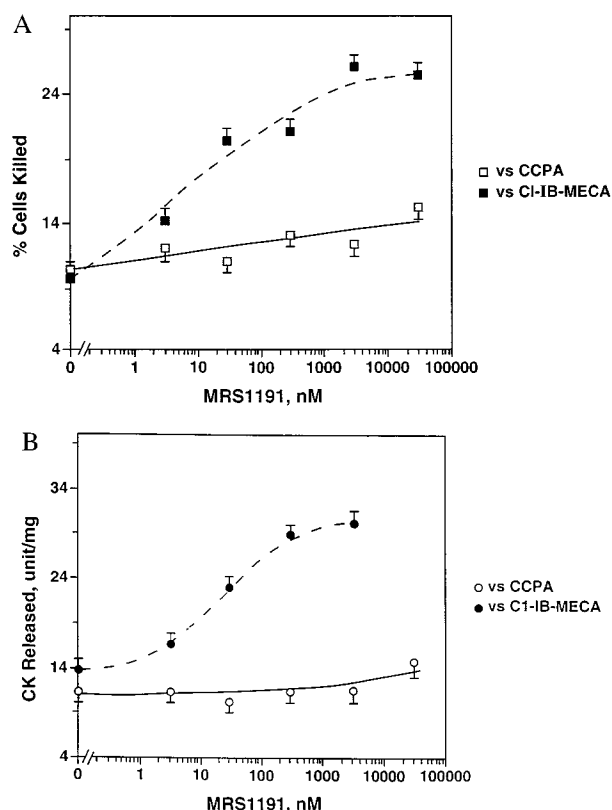


FIG. 2. Effect of MRS1191 on the CCPA- and CI-IB-MECA-induced cardioprotective effect. Cultured ventricular cells were prepared and exposed to either CCPA (10 nM) or CI-IB-MECA (10 nM) in the presence and absence of varying concentrations of the adenosine A₃ receptor antagonist MRS1191. Media were replaced with fresh media lacking the agonist or the antagonist. Cells were then exposed to normal O₂ for 10 min before being exposed to 90 min of simulated ischemia. The percentage of myocytes killed (A) and the amount of creatine kinase released (B) were determined after the 90-min ischemic period. Data represented the means and standard errors of four experiments.

also blocked the protective effect of the brief ischemia in a monophasic manner (not shown).

A Physiological Function of the Adenosine A₃ Receptor: Sustained Cardioprotection. One possible difference in the cardioprotective role of adenosine A₁ and A₃ receptors is the duration of protection mediated by the two receptors. After an initial 5-min exposure to 10 nM CCPA, concentration that produced maximal protection, the cardioprotective effect dissipated within 30 min (Fig. 4). However, the A₃ receptor-mediated protection persisted for at least 45 min after the initial exposure to an A₃ receptor agonist. Thus, the A₁ receptor-mediated protective effect is significantly shorter than that mediated by the A₃ receptor.

To provide further evidence for the notion that the A₃ receptor can mediate a sustained cardioprotective effect, atrial cells cultured from chicken embryos of the same age (14 days *in ovo*) were used. Atrial cardiac cells expressed a very low level of native A₃ receptors, as manifested by only a minimal A₃ agonist-mediated inhibition of isoproterenol-stimulated cyclic AMP accumulation (Fig. 5A). Consistent with the low level of native atrial A₃ receptor, atrial cells exhibit a shorter duration of cardioprotection than do ventricular cells (data not shown). Atrial cells were transfected with cDNA encoding the human adenosine A₃ receptor. Cells transfected with the human A₃ receptor cDNA exhibited a much more pronounced inhibition of the cyclic AMP accumulation by A₃ agonist, compared with untransfected cells or cells transfected with the vector alone

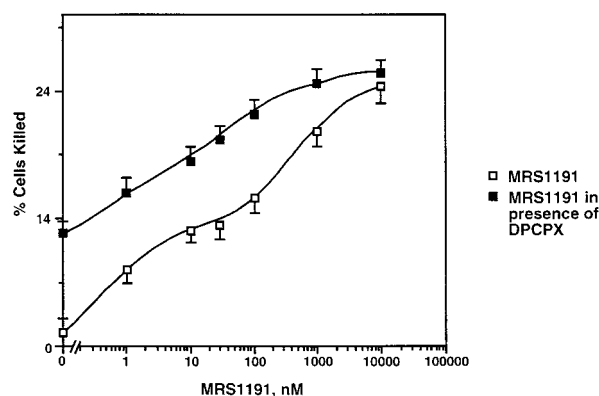


FIG. 3. Adenosine A₁ and A₃ receptors mediate the cardioprotective effect of brief ischemia. Cultured ventricular cells were exposed to 5 min of simulated ischemia in the presence and the absence of various concentrations of MRS1191 or MRS1191 plus DPCPX (1 μ M). Media were replaced by that lacking the receptor antagonists for 10 min before a 90-min ischemic exposure. The percentage of cells killed determined during the 90 min of ischemia represented the maximal level of ischemia-induced injury. Data were plotted as percentage of cells killed versus the concentration of MRS1191 in the presence and the absence of DPCPX. Data were means and standard errors of four experiments.

(one-way ANOVA and *t* test, $P < 0.01$) (Fig. 5A). These data indicate an expression of the human A₃ receptor cDNA into a functional receptor. Prior exposure to an A₃ agonist in A₃ receptor-transfected atrial cells caused a much greater decrease in the cell injury during the subsequent prolonged ischemia compared with untransfected cells or cells transfected with the vector (Fig. 5B). The percentage of decrease in the number of cells killed and the amount of CK released was $72 \pm 3\%$ and $68 \pm 5\%$, respectively, in pcDNA3/hA₃R-transfected cells versus $24 \pm 4\%$ and $26 \pm 3\%$ in untransfected cells and $28 \pm 3\%$ and $26.6 \pm 3.6\%$ in cells transfected with pcDNA3 alone (one-way ANOVA and *t* test, $P < 0.01$). The A₃ agonist-induced cardioprotection persisted even when the duration between the agonist exposure and the subsequent ischemia was increased to 30 min, which is typical of an A₃ receptor-mediated protective response.

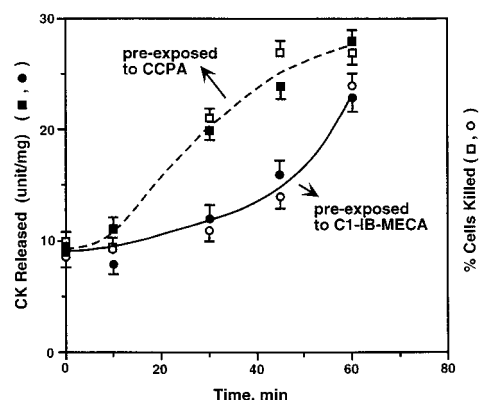


FIG. 4. Differential cardioprotective duration mediated by the adenosine A₁ and A₃ receptors. Cultured ventricular cells were prepared and exposed to either CCPA (10 nM) or CI-IB-MECA (10 nM) for 5 min. Media were replaced with fresh media lacking the agonist. Cells were then incubated under normal O₂ for 10, 30, 45, or 60 min before being exposed to 90 min of simulated ischemia. The percentage of cells killed was determined after the 90-min ischemic period. Data represented the means and standard errors of five experiments. At the 30- and 45-min time points after the adenosine agonist exposure, the percentage of cells killed and the amount of creatine kinase released were significantly less in CI-IB-MECA-treated than in CCPA-treated myocytes (*t* test, $P < 0.01$).

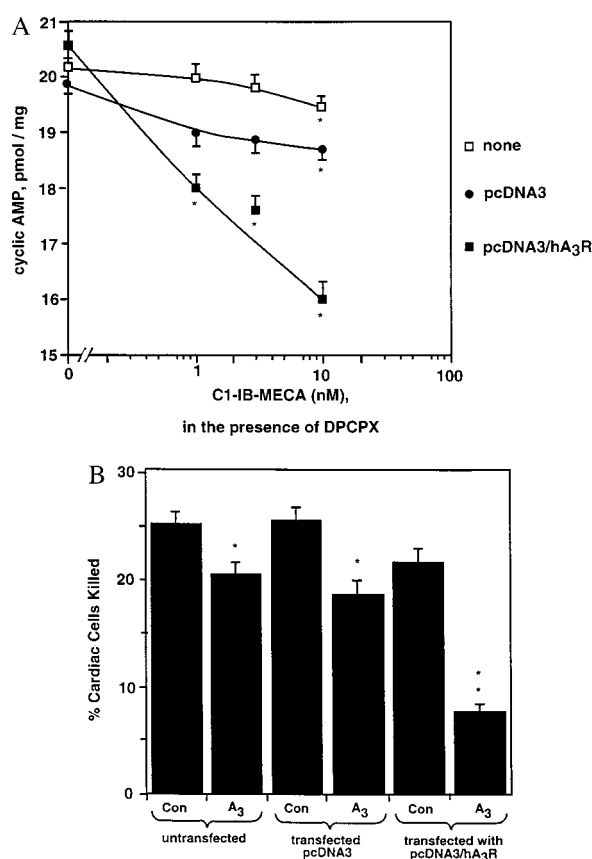


FIG. 5. Acquisition of adenosine A₃ receptor-mediated cyclic AMP and cardioprotective responses. Atrial cells were cultured from chicken embryos 14 days *in ovo* and transfected with pcDNA3 or with pcDNA3/hA₃R (full-length cDNA encoding human adenosine A₃ receptor subcloned in pcDNA3). (A) The ability of A₃ receptor agonist CI-IB-MECA to mediate inhibition of isoproterenol-stimulated cyclic AMP accumulation in untransfected atrial cells, in cells transfected with pcDNA3, or in cells transfected with pcDNA3/hA₃R was determined. DPCPX (1 μ M) was included to block the A₁ receptor. Data were means and standard errors of triplicate determinations and were typical of five other experiments. *, The cyclic AMP level was significantly less than that obtained in cells not exposed to CI-IB-MECA (control cells). At 10 nM of CI-IB-MECA, the A₃ receptor-mediated inhibition of the cyclic AMP level, expressed as percentage decrease from control cells, was significantly more in pcDNA3/hA₃R-transfected cells than in pcDNA3-transfected cells or untransfected cells (one-way ANOVA and *t* test, $P < 0.01$). (B) The ability of CI-IB-MECA to cause cardioprotection in untransfected atrial cells, in cells transfected with pcDNA3, or in cells transfected with pcDNA3/hA₃R was determined. Cells were exposed to 10 nM of CI-IB-MECA and 1 μ M of DPCPX for 5 min. Media were replaced with fresh media lacking CI-IB-MECA or DPCPX. Cells were then incubated under room air for 30 min before being exposed to 90 min of simulated ischemia. The percentage of myocytes killed and the amount of creatine kinase released (data not shown) were determined after the 90-min ischemic period. Data were the means and standard errors of seven experiments. In untransfected cells or in cells transfected with pcDNA3 or pcDNA3/hA₃R, prior A₃ agonist stimulation reduced the percentage of cells killed or the amount of CK released compared with cells not preexposed to A₃ agonist (control cells) (*, *t* test, $P < 0.05$). However, the A₃ receptor-mediated reduction in the number of cells killed or the amount of CK released, expressed as percentage of decrease from those obtained in the control cells, was significantly more in pcDNA3/hA₃R-transfected than in pcDNA3-transfected cells or untransfected cells (**, one-way ANOVA and *t* test, $P < 0.01$).

DISCUSSION

Although adenosine can exert a potent cardioprotective effect in the heart, the role of different adenosine receptors in

mediating this important process is not well understood. Activation of the adenosine A₁ and the A₃ receptors has been implicated in causing cardioprotection. However, each receptor may serve a distinct cardioprotective role in the heart. To test this hypothesis, it was necessary first to delineate clearly the effect mediated by each receptor. To accomplish this objective, agonists and antagonists selective at each adenosine receptor (19), previously characterized in the cultured chicken cardiac cells (5, 10), were employed. The cardioprotective effect of A₃ agonist CI-IB-MECA was abolished by the A₃ receptor-selective antagonist MRS1191 or MRS1097 and was not affected by the A₁-selective antagonist DPCPX. By the same token, the protective effect of A₁ agonist was abolished by the A₁ antagonist but not by the A₃ antagonist. Thus, the effect of A₁ or A₃ agonist is mediated solely by the adenosine A₁ or A₃ receptor, respectively. Activation of each receptor during a brief exposure to its agonist conferred protection against the deleterious effect of subsequent ischemia.

Because a brief ischemic exposure also can protect the myocytes against subsequent ischemia-induced injury, the question arises regarding the role of adenosine A₁ and A₃ receptors in mediating the cardioprotective effect of the brief ischemia. With the selectivity of antagonist at each receptor defined, the study determined the contribution of each receptor to the cardioprotective effect of the brief ischemia. The A₃ antagonist MRS1191 blocked the protective effect of brief ischemia in a biphasic manner. The combined presence of the A₁ antagonist DPCPX and MRS1191 converted the biphasic dose inhibition, which was obtained in the presence of MRS1191, into a monophasic curve. Whereas DPCPX was only able to inhibit partially the protective effect of brief ischemia (5), the two antagonists completely abolished the protection. Together, these data provide conclusive evidence that both adenosine receptors mediate the salutary effect of endogenous adenosine released during the initial ischemia.

Pharmacological characterization using receptor-selective agonists and antagonists also enabled delineation of potentially different cardioprotective functions mediated by each adenosine receptor. One possible difference is the duration of protection mediated by the two receptors. Whereas the A₁ receptor-mediated protection dissipated in less than 30 min, the A₃ agonist-induced effect persisted for at least 45 min after the initial agonist exposure. Therefore, although both receptors can mediate similar maximum cardioprotection, the A₁ effect is significantly shorter than the A₃ effect. Finally, that atrial myocytes transfected with exogenous A₃ receptor cDNA acquire a prolonged protective effect provides further evidence for a sustained cardioprotective role of the A₃ receptor. Thus, significant differences exist in the function served by the two receptors.

Overall, the present study has defined a physiological role of a newly identified cardiac adenosine A₃ receptor. The study indicates different cardioprotective functions mediated by the cardiac A₁ vs. A₃ adenosine receptors and provides conclusive evidence that both receptors mediate the protective effect of endogenous adenosine. The studies should have significant implications in the treatment of ischemic heart disease. Agonists selective at the two cardiac adenosine receptors may represent different therapeutic targets. A₃ receptor agonists as highly potent therapeutic agents may be free from the bradycardic and hypotensive side effects characteristic of other adenosine agonists. The ability of adenosine A₃ receptor cDNA, upon successful gene transfer, to confer cardioprotection suggests that the A₃ receptor itself can be a novel protective agent not only in the heart but also in other tissues.

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